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HISTOCOMPATIBILITY RESPONSES IN *VERONGIA* SPECIES (DEMOSPONGIAE): IMPLICATIONS OF IMMUNOLOGICAL STUDIES

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Abstract

The results of immunological analyses are correlated to those of *in situ* grafting experiments in the marine demosponge, *Verongia longissima*. The use of agglutination and cross-absorption techniques substantiate the existence of self recognition and perhaps strain-specificity in this sponge species. This specificity is consistent with previously documented clonal or strain-related patterns identified in grafting experiments.

Introduction

Since the illustrious studies of Wilson (1907) on the phenomenon of species specific aggregation in the lower Metazoa, interest in sponges has focused on cell reaggregation (Galstoff, 1925; Curtis, 1962; MacLennan, 1974). Van de Vyver (1970) was the first to record intraspecific incompatibility in the Porifera. She concluded that local populations of the freshwater sponge, *Ephydatia fluviatilis*, and the marine sponge, *Crambe crambe*, consist of a number of strain types. Each strain was defined by tissue contact incompatibility with other members of the same species in contact zones, creating a discrete border or zone of non-coalescence separating the allogeneic individuals while members of the same strain fused compatibly.

Until the 1970's the possession of an immune system was considered to be a vertebrate trait; invertebrate defense systems were considered to rely on phagocytosis and show only a crude specificity (Manning and Turner, 1976). More recently, tissue transplantation studies on the Porifera have focused on the immunorecognition and adaptive immune responses present at this lower phylogenetic level (Evans *et al.*, 1980; Hildemann *et al.*, 1980; Kaye and Ortiz, 1981; Bigger *et al.*, 1982; Curtis *et al.*, 1982; Buscema and Van de Vyver, 1983, 1984; Johnston and Hildemann, 1983; Van de Vyver, 1983; Van de Vyver and Barbieux, 1983; Neigel and Schmahl, 1984). Variability in the results of these tissue grafting studies and the diversity of histoincompatibility behavior they have demonstrated, indicate the present impracticality of formulating predictions concerning immune reactions in still uninvestigated sponge species.

The existence of strain-specificity in the marine sponge, *Verongia longissima*, has been suggested (Kaye and Ortiz, 1981) although clonal relationships for this population (Neigel and Avise, 1983) cannot be discounted. The present study investigates the possibility of employing immunological techniques such as agglutination, cross-absorption, and immunofluorescence in substantiation of apparent allogeneic incompatibility in this sponge.

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Materials and Methods

All field work and some preliminary experiments were conducted at the Bellairs Research Institute of McGill University, Barbados. Further experiments and final analyses were performed at McGill University, Montréal.

The marine demosponge, *Verongia* (=*Aplysina*) *longissima* (Carter, 1882), was the primary species studied in this investigation, and to a lesser extent the closely related species, *V. cauliformis* (Carter, 1882). *V. fistularis* (Pallas, 1766) was also observed and used in several of the experiments.

The collection area selected for this study was the same site used for previous grafting experiments (Kaye and Ortiz, 1981). Eighteen tissue samples were collected for immunological analyses. Branches of test specimens were cut from the donor and transferred to plastic bags underwater and immediately returned to the laboratory.

Preparation of antigens

Specimens were dissociated according to the procedure of Humphreys *et al.*, 1960. Cell counts of suspensions were taken with a Neubauer haemocytometer. Merthiolate was added to each suspension to retard bacterial growth. Six of the suspensions were divided into 0.1 ml aliquots for immunization, and 10 ml aliquots for agglutination and cross-absorption tests. The remaining 12 suspensions were used only for the tests.

Immunization

Six young adult rabbits (4 to 6 kg) were injected subcutaneously with 0.1 ml of the antigen emulsified with 0.2 ml of Freund's complete adjuvant on days 1, 3, 5, 7, 9, 17, 19, 21, and 23. Blood (100 ml) was collected via cardiac puncture on day 31. Sera were frozen in 5 ml aliquots for use in the agglutination and cross-absorption tests.

Agglutination tests

Reaction wells of microtitre plates contained 25λ of diluted antisera (serially diluted by doubling dilutions of calcium-magnesium-free sea water (CMF-SW) and antiserum) and 25λ of diluted antigen (1:1 with CMF-SW). Control wells contained only 25λ of diluted antigen and 25λ of CMF-SW. Plates were incubated at room temperature for 8 hours and then macroscopic and microscopic observations were recorded.

Cross-absorption tests

These tests were performed on two of the antisera to confirm the agglutination tests. Varying cell numbers of each of several antigens were spun down at 7000 rpm. The pellets were washed once in CMF-SW, resuspended, and spun down again. The pellets were then mixed with 0.3 ml of the antiserum, incubated at room temperature for 1 hour, and spun down. Agglutination tests were performed as before testing the supernatant (absorbed antiserum) against its homologous antigen. Immunofluorescence tests were confounded by autofluorescence of the sponge cells.

Results

Agglutination tests

A positive reaction (agglutination) was scored if the wells containing antiserum and antigen were cloudy and showed no pellet formation such as occurred in control

wells containing antigen only. For microscopic analyses a positive reaction was scored if sponge cells had formed aggregates which had not been disturbed during gentle mixing, and were not observed in the generally even distribution of cells in the controls.

Table I presents the results of quantitative analyses to determine agglutinating antibody content of the antisera. The antisera titres represent the highest dilution of antisera that showed agglutination. These results indicate that antisera 2 and 12 reacted with antigens 2, 11, and 12 (high titre values in antisera columns). These two antisera are therefore likely to have antibodies of similar specificity as are antisera 71 and 73 which both reacted with antigens 71, 73, 75, and 23. Antiserum 31 reacted with antigens 31, 33, and 35; and antiserum 89 reacted with only its homologous antigen, 89.

Previous work on grafting (Kaye and Ortiz, 1981) found that sponges 2, 11, and 12 were tissue-compatible and thus of the same strain of *V. longissima;* sponges 71, 73, 75, and 23 were all of the same strain of *V. longissima;* sponges 31, 33, and 35 were of the same strain, all members of *V. cauliformis;* and sponge 89 was the only sponge of strain type 15 of *V. longissima.* Thus agglutination results conform to the pattern of recognition derived from grafting studies, supporting the concept of specificity at population, strain, or individual genotype levels.

TABLE I

Comparison of agglutination results in three species of Verongia

		Antisera titers					
		V. longissima					*V. cauliformis*
Antigen	Antigen cell counts ($\times 10^6$/ml)	2	12	71	73	89	31
		V. longissima					
2	259.6	32	32	1	1	1	1
11	253.4	32	32	1	1	1	1
12	203.2	32	32	1	1	1	1
23	147.4	2	2	32	32	1	1
71	160.0	2	2	32	32	1	1
73	177.8	2	2	32	32	1	1
75	162.4	2	2	32	32	1	1
89	210.6	1	1	0	0	64	0
19	164.4	1	1	1	1	0	1
20	168.2	2	2	0	0	0	2
44	161.2	1	1	1	1	1	1
67	149.8	1	1	0	0	0	0
77	213.2	0	0	0	0	0	0
87	215.4	2	2	2	2	2	2
		V. cauliformis					
31	194.4	1	1	0	0	0	32
33	177.0	1	1	0	0	0	32
35	190.8	1	1	0	0	0	32
		V. fistularis					
TA	281.8	0	0	0	0	0	1

Cross-absorption tests

These tests were performed on antisera 71 and 12, and the results are represented in Figures 1A and 1B. These tests involved absorbing-out an antiserum by incubating various cell numbers of an antigen with the antiserum. When the antigen cell concentrations were plotted against the antiserum titres an absorption curve was obtained for the homologous antigen (Fig. 1A, #71; and Fig. 1B, #12). These curves were used as controls to compare the efficiency of the other antigens to absorb the antibodies.

The results of the cross-absorption tests for sponge antiserum 71 (Fig. 1A) dem-

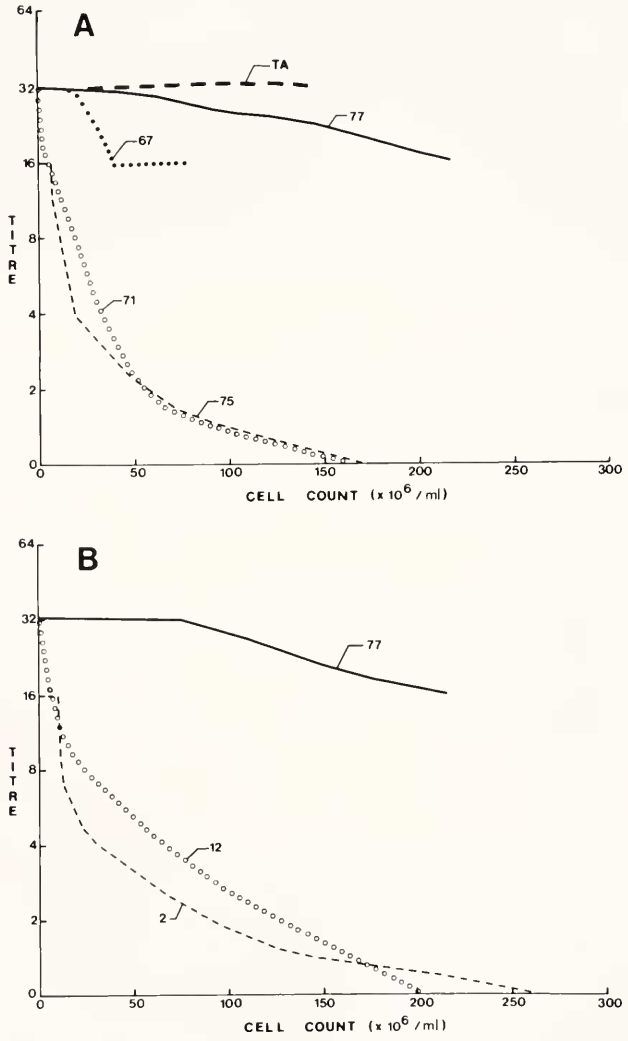

FIGURE 1. A. Results of agglutination test after absorption of α 71 by various sponge cell suspensions. The reciprocal of the dilution (TITRE axis) and concentration of sponge cell suspension (CELL COUNT axis) causing an agglutination reaction to occur are shown. Specimen TA is *Verongia fistularis*, 67 nd 77 are *V. longissima*, and 71 and 75 are the same strain (type 3) of *V. longissima*.

B. Results of agglutination test after absorption of α 12 by various sponge cell suspensions. The reciprocal of the dilution (TITRE axis) and concentration of sponge cell suspension (CELL COUNT axis) causing an agglutination reaction to occur are shown. Specimen 77 is *V. longissima*, and 2 and 12 are the same strain (type 2) of *V. longissima*.

onstrate that sponges 71 and 75 are similar and for sponge antiserum 12 (Fig. 1B) demonstrate that sponges 12 and 2 are also similar.

Discussion

Grafting experiments have provided evidence for the occurrence of strain-specificity in some sponge populations (Van de Vyver, 1970; Curtis, 1979; Evans *et al.*, 1980; Kaye and Ortiz, 1981). The allogeneic incompatibility demonstrated in these experiments is the expression of defense mechanisms that ultimately preserve the genetic integrity of the individual.

In the present study the results of the agglutination and cross-absorption tests exhibit antigenic specificities and these specificities are directly related to strain designations based upon earlier grafting experiments (Kaye and Ortiz, 1981), although clonal identity by fragmentation cannot be ruled out (Neigel and Avise, 1983). The low agglutination titres often observed with heterologous antigens from different sponge strains could be explained by: (1) the sponge cells showing antigenic cross-reactions, (2) the cells having surface components common for all strains but each strain having different levels of each of these components, and (3) the antiserum having antibodies against surface components for species and some for strain.

The agglutination test is somewhat insensitive, and a more detailed analysis is required to elucidate the type of recognition that is occurring at the low agglutination titres using other immunological techniques. Therefore, the cross-absorption test was employed. This test demonstrates the ability of same strain antigens to completely absorb the antiserum, same species antigens to absorb very little antiserum, and different species antigens to absorb none of the antiserum. It is apparent from these cross-absorption tests that the antigens have qualitative differences. In other words, the type of recognition that is occurring at the low agglutination titres is more likely to be due to a species recognition and not the results of cross reactivity or quantitative differences in the surface components of the sponge cells. However, the fact that a small amount of antibody was absorbed by heterologous sponge cells may indicate that some antibodies were formed against components common to the species. Nevertheless, it appears that surface components on the cells of *V. longissima* responsible for strain recognition in this sponge play the dominant role in eliciting antibody formation in rabbits.

In light of reaggregation and grafting studies that have demonstrated species and presumed strain-specific recognition in sponges, it seems that allogeneic effects may have been missed by other workers in reaggregation studies (Spiegel, 1954; MacLennan and Dodd, 1967) because factors were isolated from bulk sponge material, possibly derived from multiple allogeneic individuals. This problem may require the reinterpretation of these results, and should certainly be considered in future studies.

The limited supply of antigens and antisera restricted the extent to which the agglutination and cross-absorption tests could be carried out. To allow for immunological results to be employed as conclusive evidence regarding histocompatibility among specimens of *V. longissima* the tests should be carried out for all the sponges employed in the grafting experiments (Kaye and Ortiz, 1981). However, sufficient data has been presented to demonstrate the possibilities of employing immunological methods to show the occurrence of compatibility between individual specimens of *V. longissima*, whether of strain type or clonal basis. Once the results of these techniques are extensively compared to grafting results, and if the results consistently agree, these immunological methods can be employed to gain conclusive evidence regarding strain specificity in other sponges.

This study offers another technique that could be extended to future research in this area which might provide insight into the mechanisms involved in histocom-

patibility reactions occurring in allogeneic rejections, and in the exquisite immune system that these lower invertebrates possess.

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